ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2006, p. 382–384 Vol. 50, No. 1
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.1.382–384.2006

Arginine or Nitrate Enhances Antibiotic Susceptibility of *Pseudomonas aeruginosa* in Biofilms

Giorgia Borriello,[1] Lee Richards,[1] Garth D. Ehrlich,[2] and Philip S. Stewart[1]*

Center for Biofilm Engineering and Department of Chemical Engineering, Montana State University—Bozeman, Bozeman, Montana 59717-3980,[1] and Center for Genomic Sciences, Allegheny Singer Research Institute, 320 E. North Ave., Pittsburgh, Pennsylvania 15212[2]

Received 1 June 2005/Returned for modification 1 July 2005/Accepted 10 October 2005

Arginine enhanced the killing of *Pseudomonas aeruginosa* by ciprofloxacin and tobramycin under anaerobic, but not aerobic, growth conditions. Arginine or nitrate also enhanced the killing by these antibiotics in mature biofilms, reducing viable cell counts by a factor of 10 to 100 beyond that achieved by antibiotics alone.

The opportunistic bacterial pathogen *Pseudomonas aeruginosa* is responsible for persistent infections such as those associated with cystic fibrosis (CF) lung disease, burn wounds, otorrhea, and the cornea (2). One of the factors contributing to the recalcitrant nature of these infections is that the bacteria form biofilms in which they are protected from killing by antimicrobial agents. This protection does not depend on the poor penetration of antibiotics into the biofilm (11). Rather, it appears that the tolerance of these bacteria to antibiotics is tied to physiological or phenotypic changes in a subset of the cells in the biofilm (1, 5, 7, 9, 11, 14). A particularly powerful mechanism suggests that biofilms contain anoxic regions where the metabolic activity, and also the susceptibility to antibiotics, of aerobes such as *P. aeruginosa* is diminished.

The common occurrence of oxygen gradients in biofilms is well known, and in vitro measurements with oxygen microelectrodes have demonstrated the presence of anoxic regions within *P. aeruginosa* biofilms in particular (11, 12, 15). One in vivo study has also revealed the presence of anaerobic pockets in the infected mucus which lines the airways of individuals with CF (13), and another has provided molecular evidence for bacterial anaerobiosis in CF lung disease (16). In vitro investigations have furthermore demonstrated that *P. aeruginosa* biofilms commonly exhibit stratified patterns of metabolic activity. Protein synthesis, for example, is very active in regions of the biofilm adjacent to an oxygen source, with very limited activity detectable in the anoxic zones (11, 12, 15).

These observations support the following cascade of effects in a *P. aeruginosa* biofilm: (i) oxygen limitation leads to anoxic regions in the biofilm; (ii) in anoxic regions, metabolic activity slows down or stops; and (iii) metabolically inactive bacteria are less susceptible to killing by antibiotics. There is some experimental evidence in support of the third conjecture (1).

If the recalcitrance of *P. aeruginosa* biofilm infections depends on the occurrence of anoxic regions in the biofilms where the bacteria are metabolically quiescent, then one way to improve antibiotic chemotherapy for such infections is to stimulate anaerobic metabolism. In the case of *P. aeruginosa*, this can be accomplished by either adding an electron acceptor that permits denitrification or by adding arginine, which *P. aeruginosa* can ferment (3, 10). This article reports the results of an in vitro investigation on the effects of arginine and nitrate on the susceptibility of *P. aeruginosa* in biofilms to ciprofloxacin and tobramycin.

The general strategy of feeding a biofilm during antibiotic treatment is one that has not been experimentally tested. The only example of this type of test that we are aware of is a demonstration that increasing the provision of nutrients during exposure to rifampin increases the killing of *Staphylococcus epidermidis* in biofilms (17).

Bacterial susceptibility to either 1 μg/ml ciprofloxacin or 10 μg/ml tobramycin was measured in colony biofilms as described previously (1). Ciprofloxacin targets DNA gyrase and interferes with DNA synthesis, while tobramycin binds to the ribosome and interferes with protein synthesis. A 50-μl drop of a planktonic culture of *P. aeruginosa* strain PAO1 was deposited on a 25-mm-diameter, 0.2-μm polycarbonate membrane resting on a tryptic soy agar (TSA) plate. The plates were incubated for 4 h at 37°C. These young colony biofilms were then transferred to TSA plates supplemented with antibiotic, TSA plates supplemented with antibiotic plus 0.4% L-arginine, or simply unsupplemented TSA plates. This concentration of arginine is the same as that used in a medium for growing *P. aeruginosa* in a previous study (7). The biofilms were then incubated for an additional 12 h at 37°C. Some colony biofilms were incubated under anaerobic conditions for the 12-h test period by placing the plates in an anaerobic gas bag. Viable cells were enumerated by serial dilution and drop plating.

Arginine supplementation did not affect the number of bacteria over the 12-h test period under either aerobic or anaerobic growth conditions. The number of viable cells measured after 16 h of total incubation time was not statistically different for biofilms grown with arginine than for biofilms grown without arginine ($P = 0.40$ and 0.63 under aerobic and anaerobic conditions, respectively). This suggests that arginine does not affect bacterial replication. Higher concentrations of arginine also did not detectably enhance growth (data not shown).

Under aerobic conditions, the antibiotic efficacy against 4-h colony biofilms was not affected by the presence of arginine (Fig. 1A). In contrast, arginine enhanced antibiotic efficacy

* Corresponding author. Mailing address: Center for Biofilm Engineering, Montana State University—Bozeman, 409 Cobleigh Hall, Bozeman, MT 59717-3980. Phone: (406) 994-4770. Fax: (406) 994-6098. E-mail: phil_s@erc.montana.edu.

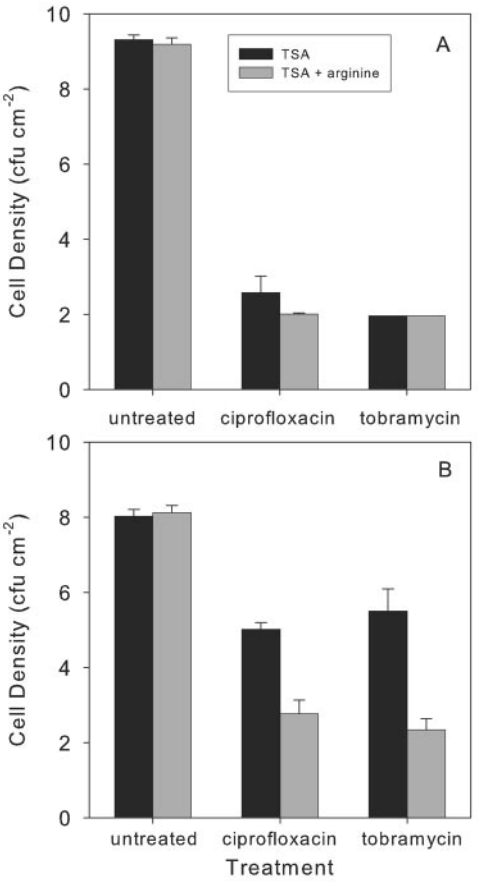

FIG. 1. Effect of arginine on the antibiotic susceptibility of *P. aeruginosa* in young (4-h) colony biofilms under aerobic (A) and anaerobic (B) conditions. Error bars denote standard deviations.

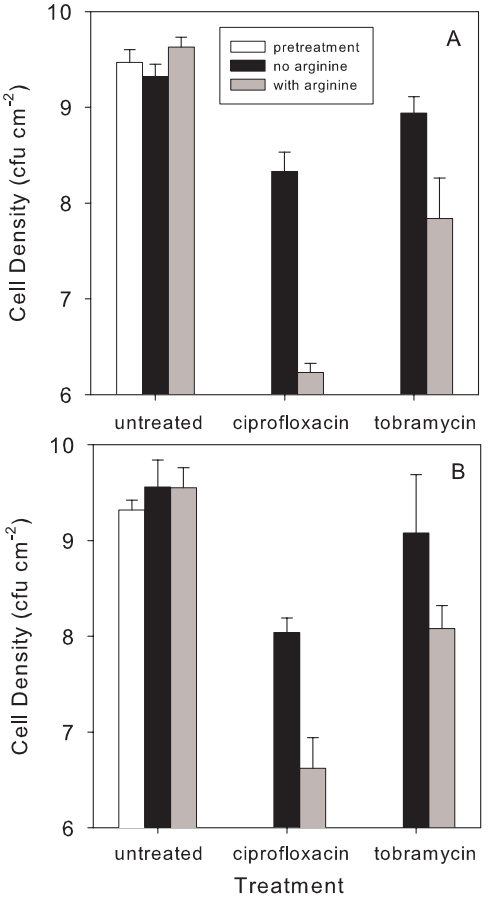

FIG. 2. Effect of arginine on the antibiotic susceptibility of *P. aeruginosa* in aerobic 48-h colony biofilms (A) and 72-h drip flow reactor biofilms (B). Error bars denote standard deviations.

against 4-h colony biofilms under anaerobic conditions (Fig. 1B). In the absence of oxygen and without arginine, the log reductions realized after 12 h of treatment with ciprofloxacin and tobramycin were 2.61 ± 0.13 and 2.14 ± 0.42, respectively. In the absence of oxygen and the presence of arginine, the log reductions for these two antibiotics rose to 4.86 ± 0.25 and 5.29 ± 0.21, respectively. These differences were statistically significant based on a two-sided *t* test ($P = 0.0014$ and 0.0026 for ciprofloxacin and tobramycin, respectively). These results suggest that even though arginine was not a factor in promoting cell division, it was able to enhance antibiotic susceptibility under anaerobic conditions, presumably by increasing the metabolic rate.

The effect of arginine on *P. aeruginosa*'s susceptibility to ciprofloxacin and tobramycin was also investigated using mature biofilms from two different growth systems. We deliberately used two different biofilm models with quite different media. The rationale for this method is that if the enhancement of antibiotic action by arginine supplementation is robust, then this enhancement will be observed regardless of the details of the growth conditions. Susceptibility was evaluated against 48-h colony biofilms and against biofilms grown for 72 h in a continuous drip flow reactor system (15). Colony biofilms were grown on TSA for 48 h prior to a 12-h antibiotic exposure. Colony biofilms are dense aggregates of bacteria, developed in the absence of fluid flow, on top of filter membranes resting on agar plates. Drip flow biofilms were grown on a glucose-minimal medium at 37°C for 72 h prior to treatment for 12 h with the antibiotics in the same medium. Drip flow biofilms develop in a low-fluid shear environment on stainless steel slides over which the medium flows in a thin film. Details of these methods are provided elsewhere (1, 15).

For mature biofilms, it can be anticipated that a fraction of the biofilm will be anoxic. Mature biofilms should therefore reflect, at least in part, the behavior of bacteria under anaerobic conditions.

Arginine supplementation increased biofilm susceptibility to both ciprofloxacin and tobramycin in both of the systems tested (Fig. 2). Mature (48-h) colony biofilms were highly recalcitrant to 12-h exposures of these antibiotics in the absence of added arginine; log reductions were 1.13 ± 0.14 and 0.52 ± 0.12 for ciprofloxacin and tobramycin, respectively. These log reductions rose to 3.23 ± 0.06 and 1.63 ± 0.30, respectively, when the TSA was supplemented with 0.4% arginine. This enhancement of killing by arginine was statistically significant ($P = 0.0002$ and 0.026 for ciprofloxacin and tobramycin, respectively). In the drip flow reactor system, log reductions increased from 1.28 ± 0.13 and 0.35 ± 0.24 for ciprofloxacin and

TABLE 1. Summary of enhancement of antibiotic efficacy against *P. aeruginosa* colony biofilms by added arginine, nitrate, or nitrite

Antibiotic	Supplement	Efficacy against *P. aeruginosa*[a]	
		Log reduction	Log difference
Ciprofloxacin	None	1.13 ± 0.14	
Ciprofloxacin	Arginine	3.23 ± 0.06	2.1
Ciprofloxacin	Nitrate	3.19 ± 0.19	2.1
Ciprofloxacin	Nitrite	3.04 ± 0.20	1.9
Tobramycin	None	0.52 ± 0.12	
Tobramycin	Arginine	1.63 ± 0.30	1.1
Tobramycin	Nitrate	1.90 ± 0.45	1.4
Tobramycin	Nitrite	1.24 ± 0.06	0.7

[a] The log difference is the log reduction measured for antibiotic with arginine or nitrate less the log reduction for antibiotic alone. A log difference of 1 corresponds to a 10-fold difference in viable cell numbers; a log difference of 2 corresponds to a 100-fold difference in viable cell numbers.

tobramycin, respectively, to 2.73 ± 0.39 without arginine and 1.27 ± 0.29 in the presence of added arginine. In both cases, the enhancement of antibiotic action by arginine was statistically significant ($P < 0.04$). In neither system did the addition of arginine lead to bacterial replication in the absence of antibiotic treatment. The differences in log reductions reported above correspond to approximately 10 to 100 times fewer viable cells in the biofilms treated with antibiotic plus arginine than in the biofilms treated with antibiotic alone (Table 1).

P. aeruginosa can use nitrate as an alternate electron acceptor in the absence of oxygen, so we hypothesized that provision of nitrate during antibiotic exposure would also enhance efficacy. TSA was supplemented with either 1% potassium nitrate or 1% potassium nitrite. Colony biofilms were grown for 48 h on TSA in the absence of nitrate or nitrite and then transferred to antibiotic-containing TSA plates supplemented with either 1% potassium nitrate or 1% potassium nitrite for 12 h. The addition of nitrate or nitrite enhanced killing by ciprofloxacin and tobramycin by a factor similar to that measured for arginine (Table 1). Exposure to nitrate or nitrite for 12 h in the absence of antibiotic had no effect on viable cell numbers. In a previous study, we reported that nitrate supplementation had little effect on, or even decreased, the antibiotic susceptibility of *P. aeruginosa* in biofilms (1). These earlier experiments were performed using young (4-h) colony biofilms rather than the mature (48-h) colony biofilms used to collect the data reported in this article. This discrepancy suggests that the effects of nitrate on antibiotic susceptibility are modulated by biofilm age.

These data indicate that arginine or nitrate can increase the susceptibility of *P. aeruginosa* to two classes of antimicrobials in mature biofilms. *P. aeruginosa* colony biofilms (11, 12) and drip flow biofilms (15) have both been shown to harbor regions of very low oxygen concentration. We hypothesize that it is in these anoxic regions where bacteria are poorly killed due to very low metabolic rates. We further speculate that arginine or nitrate positively modulate the antibiotic susceptibility of biofilms by increasing the metabolic activity of bacteria in the anaerobic zones of mature biofilms.

Our results support the possibility of using arginine as an adjuvant to enhance the efficacy of antimicrobial chemotherapy when a pseudomonal biofilm is implicated. Interestingly, arginine therapy has been suggested and tested in the context of CF (4, 6, 8). In these studies, arginine was evaluated for its mucolytic activity (4, 8) and for its potential to enhance nitric oxide concentrations and thereby stimulate ciliary clearance (6). Neither of these uses is part of modern CF therapy. The past use of arginine suggests that it is safe when properly buffered (8). There does not appear to have been any in vivo evaluation of the interaction between arginine and antibiotics.

This work was supported by NIH awards 2R01DC04173 and R01GM067245-02 and by an award from the W. M. Keck Foundation.

REFERENCES

1. **Borriello, G., E. Werner, F. Roe, A. M. Kim, G. D. Ehrlich, and P. S. Stewart.** 2004. Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. Antimicrob. Agents Chemother. **48:**2659–2664.
2. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
3. **Cunin, R., N. Glansdorff, A. Piérard, and V. Stalon.** 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. **50:**314–352.
4. **Dietzsch, H.-J., B. Gottschalk, K. Heyne, W. Leupold, and P. Wunderlich.** 1975. Cystic fibrosis: comparison of two mucolytic drugs for inhalation treatment (acetylcysteine and arginine hydrochloride). Pediatrics **55:**96–100.
5. **Drenkard, E., and F. M. Ausubel.** 2002. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature **416:**740–743.
6. **Grasemann, H., S. S. Gartig, H. G. Wiesemann, H. Teschler, N. Konietzko, and F. Ratjen.** 1999. Effect of L-arginine infusion on airway NO in cystic fibrosis and primary ciliary dyskinesia syndrome. Eur. Respir. J. **13:**114–118.
7. **Mah, T. F., B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O'Toole.** 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. Nature **426:**306–310.
8. **Solomons, C. C., E. K. Cotton, R. Dubois, and M. Pinney.** 1971. The use of buffered L-arginine in the treatment of cystic fibrosis. Pediatrics **47:**384–390.
9. **Spoering, A. L., and K. Lewis.** 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J. Bacteriol. **183:**6746–6751.
10. **Vander Wauven, C., A. Piérard, M. Kley-Raymann, and D. Haas.** 1984. *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. J. Bacteriol. **160:**928–934.
11. **Walters, M. C., III, F. Roe, A. Bugnicourt, M. J. Franklin, and P. S. Stewart.** 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob. Agents Chemother. **47:**317–323.
12. **Werner, E., F. Roe, A. Bugnicourt, M. J. Franklin, A. Heydorn, S. Molin, B. Pitts, and P. S. Stewart.** 2004. Stratified growth in *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **70:**6188–6196.
13. **Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, P. Birrer, G. Bellon, J. Berger, T. Weiss, K. Botzenhart, J. R. Yankaskas, S. Randell, R. C. Boucher, and G. Döring.** 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J. Clin. Investig. **109:**317–325.
14. **Xu, K. D., G. A. McFeters, and P. S. Stewart.** 2000. Biofilm resistance to antimicrobial agents. Microbiology **146:**547–549.
15. **Xu, K. D., P. S. Stewart, F. Xia, C.-T. Huang, and G. A. McFeters.** 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. **64:**4035–4039.
16. **Yoon, S. S., R. F. Hennigan, G. M. Hilliard, U. A. Ochsner, K. Parvatiyar, M. C. Kamani, H. L. Allen, T. R. DeKievit, P. R. Gardner, U. Schwab, J. J. Rowe, B. H. Iglewski, T. R. McDermott, R. P. Mason, D. J. Wozniak, R. E. W. Hancock, M. R. Parsek, T. L. Noah, R. C. Boucher, and D. J. Hassett.** 2002. *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev. Cell **3:**593–603.
17. **Zheng, Z., and P. S. Stewart.** 2004. Growth limitation of *Staphylococcus epidermidis* in biofilms contributes to rifampin tolerance. Biofilms **1:**31–35.